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Hemolysis as a Possible Indicator of Neurotoxicity Induced by Organic Solvents

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The expense, length of time and number of animals required for routine toxicity testing have provided the incentive for finding alternative techniques which are faster, less expensive and equally valid. The purpose of this work was to examine the value of a simple *in vitro* test (hemolysis) as a correlate of the neurotoxicity produced by commonly used industrial organic solvents. Incubation of rat erythrocytes with organic alcohols produced hemolysis which correlates with the potency of the same alcohols to suppress membrane excitability, measured as reduction in the evoked action potential of the rat sciatic nerve. The hemolytic activity also reflects changes in water solubility among the compounds and thus can be used as an index of *in vivo* neurotoxicity, the extent of which partly depends on absorption of the agent and delivery to nerve tissue. Hemolysis therefore may be of value as a preliminary test for assessing the neurotoxicity of organic solvents.

Introduction

There is a pressing need to assess a large number of environmental and industrial chemicals for their potency in inducing neurotoxicity. Unlike damage to many other organ systems, neurotoxicity is a particularly serious clinical problem because neurons are highly differentiated and do not undergo cell division; thus the toxicity is often irreversible or only partially reversible. Since the traditional methods for evaluating neurotoxicity are both time-consuming and expensive, and many widely used industrial solvents are potentially neurotoxic, a new and efficient technique for first-tier neurotoxicity testing would be of considerable value. Unfortunately, there are many potential sites and mechanisms through which neurotoxic substances can act, and it would be virtually impossible for one test to be valid in all cases. However, with some preliminary knowledge of the physicochemical characteristics of the agent (which is almost always available), it should be possible to focus testing appropriately. For example, organic solvents have lipid solubility as a prominent and common feature, and one would expect *a priori* that their pharmacologic action would depend at least in part on incorporation into the cell lipid bilayer. We present data in this report demonstrating the value of one simple test of membrane action (erythrocyte hemolysis) as a predictor of the neurotoxic potency of organic solvents.

Since the demonstration by Meyer and Overton (*1–3*) that the potency of many anesthetic agents is related to lipid solubility, many reports have confirmed and extended this principle of drug action. The relationship has been used to explain the potency of local anesthetics (*4*), general anesthetics (*5*), and tranquilizers and central nervous system depressants (*6*). The Meyer-Overton rule does not explain how the drug's lipid solubility leads to a disruption of membrane excitability, but many experimental observations are consistent with this view (*6–12*).

Many workers have used the eyrthrocyte as a model for studying drug interactions with an excitable membrane. Although the appropriateness of the erythrocyte as a model has been questioned (*13*), decreased nerve excitability has been correlated with a number of drug effects in the erythrocyte membrane. For example, the critical volume at which the erythrocyte membrane undergoes osmotic hemolysis (*14*) is increased by the alcohols and by volatile general anesthetics (*15*). This effect correlates with the extent to which these agents are incorporated into the erythrocyte and produce membrane swelling (*16*). Because incorporation of these agents into the erythrocyte results in hemolysis and incorporation into the nerve membrane results in a loss of excitability, we proposed that hemolysis might be a reliable first-tier assay for screening the neurotoxic potential of organic solvents. We chose to compare the effects of the 10 primary alcohols from methanol to decanol in producing hemolysis of rat erythrocytes and

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the effects of the same agents in decreasing excitability of the rat sciatic nerve.

Methods

Male Sprague-Dawley rats were decapitated, and samples of trunk blood were placed in tubes of Alsever's buffer (0.42% sodium chloride, 0.8% sodium citrate and 2.05% glucose, adjusted to a pH of 6.0) and heparinized with 500 units of heparin/mL of Alsever's solution. The erythrocytes were washed three times in Alsever's solution and then resuspended to produce a 4% solution of erythrocytes in Alsever's. Quadruplicate samples containing 2% erythrocytes were combined with various concentrations of the alcohols in a total volume of 5 mL. The mixture was vortexed and incubated for 5 min at 0°C. The solution was then centrifuged and filtered to separate all particulate matter from the hemoglobin-containing supernatant. The free hemoglobin content of the solution was measured spectrophotometrically at 543 nm against blanks in which erythrocytes had been incubated with buffer only. All solutions were kept on ice.

Following decapitation of the rat, the sciatic nerve was quickly dissected and placed in an isolated nerve chamber with several partitions. The fluid-tight compartments were sealed with Vaseline. The nerve was stimulated and the compound action potential recorded as previously described (*17*). Control measurements were made with the central compartment containing saline. Various concentrations of alcohol–saline mixtures were then substituted, and the action potential was monitored until it reached a plateau response (usually about 30 min). The nerve action potential was then permitted to recover with fresh saline in the test compartment. Only nerve preparations which showed at least partial recovery were used in the data analysis.

Results

Figure 1 shows the dose–response data for the erythrocyte hemolysis and Figure 2 shows data for the depression of sciatic nerve activity. For each of the alcohols there was a log-linear relationship between the concentration of the alcohol and the magnitude of the response. Furthermore, with increasing length of the aliphatic chain of the alcohol, there was a progressive shift of the curves to the left, indicating that the longer chain alcohols are more potent in producing both hemolysis and decreasing nerve excitability. Interestingly, the series from methanol to 1-hexanol produced a family of curves with parallel slopes for each of the two responses. In order to determine the correlation between these two responses, the concentration of these alcohols (from methanol to 1-hexanol) which produced a 50% reduction in the nerve response and the concentration which produced a standard degree of hemolysis measured spectrophotometrically (OD = 0.3) was calculated from the best-fitting linear regression lines. Figure 3 shows a linear correlation between these two standardized values of the first six alcohols (R = 0.99). It should be noted, however, that the concentration range for the two responses is quantitatively different. For example, the concentration of methanol required for hemolysis was approximately ten times greater than the concentration which blocked the nerve action potential.

The slopes of the hemolysis dose-response curves could be grouped into two subsets: the shorter chain alcohols (from methanol to 1-hexanol) induced a hemolytic response with a steep slope, whereas 1-heptanol to 1-decanol exhibited less hemolytic activity and produced more shallow slopes. The slopes of the nerve dose-response curves were parallel for all the alcohols (except 1-nonanol), indicating a progressive increase in absolute potency with chain length. (Nerve data for

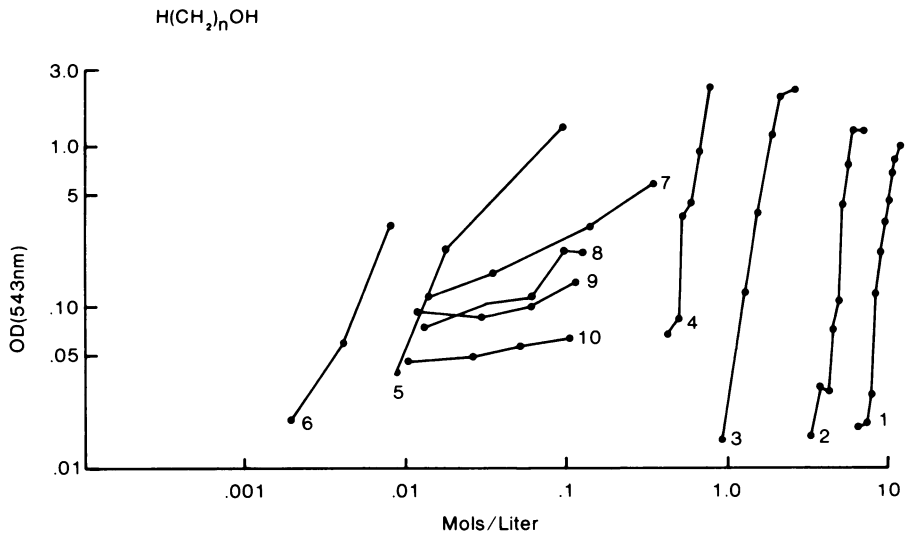

FIGURE 1. Hemolytic potency of primary alcohols (n = 1 - 10). Various concentrations of alcohols were incubated with a 2% solution of rat erythrocytes. The optical density of the supernatant was measured at 543 nm. Each point represents the mean of quadruplicate determinations.

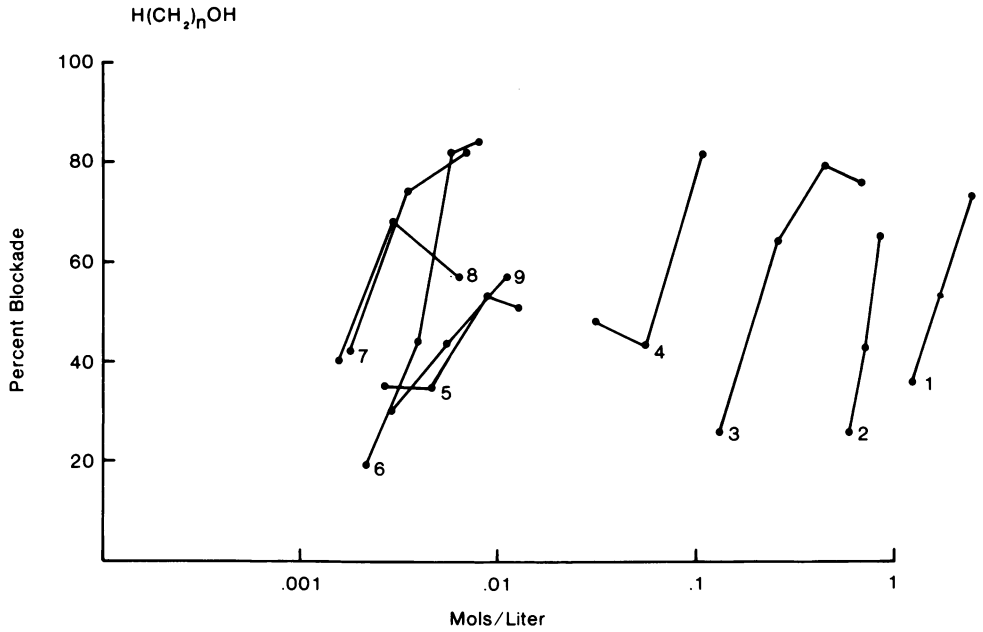

FIGURE 2. Loss of nerve excitability induced by primary alcohols (n = 1 - 9). Isolated rat sciatic nerves were exposed to various concentrations of alcohols. Loss of excitability was calculated as a percent of the pre-drug control amplitude of the compound action potential. Each point is the mean of data from at least four nerves. The numbers associated with each line indicate the aliphatic chain length of each alcohol.

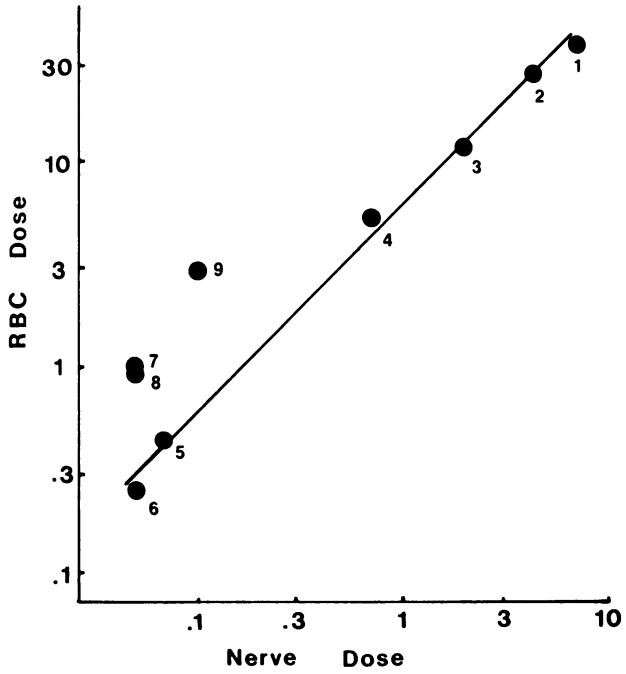

FIGURE 3. Correlation between hemolytic activity and loss of nerve excitability induced by primary alcohols (n = 1 -9). The alcohol concentration which produced a median hemolysis (defined as OD = 0.3) is plotted versus the concentration of the same alcohol which produced a 50% loss of nerve excitability. The numbers indicate the aliphatic chain length of each alcohol. The regression line was fitted using data only from the first six alcohols.

1-decanol is not included because the limited water solubility of 1-decanol prevented making aqueous concentrations greater than 1% and the loss of nerve excitability could not be reversed during the saline wash.) However, Figure 2 does not show another important feature of the nerve response. With increasing chain length, the onset of the alcohol-induced depression was delayed and the decreased excitability was more difficult to reverse by washing the nerve in saline. The longer chain alcohols were more difficult to mix in aqueous solution, which may account for the slower access to the nerve.

Discussion

These results show that hemolytic activity of the primary alcohols correlates with their ability to block nerve conduction. However, the absolute concentration required for hemolysis was usually greater than the concentration which blocked the nerve. Thus, hemolysis seems to predict the relative neurotoxicity among a group of structurally related compounds, but does not provide a measure of the solvent concentration required for neurotoxicity.

The effects of various agents on the stability of the erythrocyte membrane have been extensively studied (*1–12*). Hemolysis has been shown to correlate well with the lipid/water partition coefficient. However, there are many other factors, including perturbation of the protein structure (*13*), which seem to contribute as well. Despite many differences in the composition of nerve and erythrocyte membranes, they seem to respond similarly when exposed to a variety of alcohols.

Water solubility is a factor which will limit the absorption and distribution of the compound to its site of action in nerve membrane after *in vivo* exposure.

Thus, although the longer chain alcohols may be inherently more neurotoxic, their delivery to the nerve site of action after an environmental exposure may be severely limited. The difficulty in comparing the effects of the longer chain alcohols arises from a change in the slope of the hemolytic response. The alcohols from 1-heptanol to 1-decanol had only limited hemolytic activity and produced a second family of parallel curves with a much broader slope (Fig. 1). Previous reports of the effects of drugs on the erythrocyte membrane have not commented on this change in slope. However, in relating the structures of several homologous series of solvents to their potency as anesthetics, the term "cut-off" has been used to describe the parabolic relationship between biologic activity and chain length (*18,19*). The physicochemical property most frequently correlated with this cut-off is decreasing water solubility. The marked decrease in water solubility of the longer chain alcohols may explain why the correlation between the hemolytic and nerve effects of alcohols from 1-heptanol to 1-nonanol does not conform to the rest of the alcohol series. These observations are consistent with the view taken by others (*20,21*) that lipid solubility and water solubility should both be taken into account when characterizing the biologic activity of organic solvents. The water/organic solvent partition coefficient can provide an important clue to both the hemolytic activity and the nerve conduction blocking activity of a given agent.

The dose-response characteristics of the hemolytic response seem to take into account two important factors related to neurotoxicity: (a) among water-soluble solvents, the steep hemolytic dose-response lines correlate directly with the neurotoxicity of the same solvents; (b) the shallow sloped dose-response lines of the longer chain alcohols indicate a marked decrease in hemolytic activity. This loss of hemolytic activity is probably related to water insolubility which also will limit absorption of the agents in an *in vivo* system. Although these compounds may be inherently neurotoxic, their lack of water solubility may counter their actual neurotoxicity after *in vivo* exposure. This accounts for the loss of a correlation between the hemolysis and nerve blockade responses of the longer chain alcohols.

These data demonstrate the value of erythrocyte hemolysis as a preliminary test for assessing neurotoxicity. Solvents which are capable of disrupting erythrocytes are also capable of blocking nerve excitability. Apparently, both of these actions rely on similar mechanisms, namely, solubility of the substance in the biologic membrane. Therefore, this test should be limited to compounds such as organic solvents with significant lipid solubility. Neurotoxic substances which act through receptor proteins or other specific synaptic-mediated events would not be detected by this screen and should be examined using other neurotoxicity assays. However, since hemolysis does suggest that there may be a change in nerve membrane excitability, the erythrocyte test may be a useful first-tier screen of compounds suspected of producing a functional change in nerve membranes. Once detected by this method, more specific second- and third-tier tests could be employed to elucidate the exact nature of the neurotoxicity.

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